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Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of isopimarane diterpenes from Velloziaceae

Angelo da Cunha Pinto¹, Ricardo Vessecchi^{2*}, Carmelita Gomes da Silva³, Ana Carolina Lourenço Amorim¹, Helvécio Martins dos Santos Júnior¹, Michelle Jakeline Cunha Rezende¹, Paul J. Gates⁵, Claudia Moraes de Rezende¹, and Norberto Peporine Lopes^{4*}

¹ *Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio de Janeiro, Cidade Universitária, CT, Bloco A, sala 626A, CEP 21941-909, Rio de Janeiro - RJ, Brazil*

² *Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, 14040-901, Ribeirão Preto-SP, Brazil*

³ *Instituto Federal de Educação, Ciência e Tecnologia. Campus Nilópolis, R. LúcioTaváres, 1045, CEP 26530-060, Centro, Nilópolis-RJ, Brazil*

⁴ *Núcleo de Pesquisas em Produtos Naturais e Sintéticos (NPPNS) - Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café, s/n, CEP 14040-903, Ribeirão Preto - SP, Brazil*

⁵ *School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, United Kingdom*

Corresponding authors:

vessecchi@usp.br; npelopes@usp.br

Prof. Dr. Ricardo Vessecchi

Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, 14040-901, Ribeirão Preto-SP, Brazil

Prof. Dr. Norberto P. Lopes

Núcleo de Pesquisas em Produtos Naturais e Sintéticos (NPPNS) - Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café, s/n, CEP 14040-903, Ribeirão Preto - SP, Brazil

RATIONALE: The study of natural products by electrospray ionization tandem mass spectrometry (ESI-MS/MS) is an important strategy for the characterization of the major fragmentation reactions which can then help to determine the composition of complex mixtures. Application of ESI-MS/MS to a series of isopimarane diterpenes from Velloziaceae allowed the rationalization of their fragmentation mechanisms.

METHODS: Velloziaceae diterpenes were isolated by silica gel column chromatography and investigated by ESI-MS/MS analysis. The fragmentation studies were performed on a quadrupole-time-of-flight instrument using N₂ as the collision gas. To help rationalize the fragmentation pathways observed, the geometry and sites of reactivity of the diterpenes were obtained by theoretical calculations using the B3LYP/6-31 + G(d,p) model. Fragmentation mechanisms were proposed on the basis of the calculated protonation sites and product ions energies using density functional theory (DFT) methods.

RESULTS: The presence of hydroxyl and carbonyl groups on the terpene core influences the protonation site observed. One compound showed a radical cation as the base peak. MS/MS spectra exhibit water elimination as the major fragmentation pathway (via two ways), either when protonation takes place on the oxygen atom, or through elimination after activation from hydrogen migration. After the elimination of water, the formation of an endocyclic double bond induces a sequential retro-Diels-Alder (RDA) reaction as the major fragmentation step.

CONCLUSIONS: A thorough rational analysis of the fragmentation mechanisms of protonated Velloziaceae diterpenes was used to propose the dissociation mechanisms in ESI-MS/MS. The presence of esters in the side chain also influenced the intensity or occurrence of the observed protonated or cationized molecules in ESI-MS. These results will aid the identification of analogues in sample extracts in future metabolomics studies.

Keywords: fragmentation mechanism, Velloziaceae, natural products, terpene

Introduction

The Velloziaceae family comprises about 270 species which are distributed mainly in South America and Africa. These plants are found growing in inhospitable environments - sandy and/or rocky soils, with high solar irradiation and low amounts of water. A typical biome is found above 1000 m altitude - known as rupestrian fields¹. Despite this apparent weakness, many Velloziaceae species present a striking feature of being desiccation tolerant resurrection plants, such as species of the *Vellozia* genus and the African *Xerophyta* and *Talbotia*². This feature, along with chemical composition, contributes to the surprising longevity of these plants³. Previous phytochemical studies of *Vellozia* demonstrated the isolation of different molecular skeletons of diterpenes such as clerodane, cleisthantane, isopimarane, strobane, kaurane, halimane and labdane⁴. Over a period of approximately 25 years, Pinto and co-workers reported the isolation of around 190 diterpenes, 52 triterpenes, 5 flavonoids and 3 sterols from several studies of Brazilian Velloziaceae⁵.

The structural characterization of the isopimarane diterpenes presented in this paper was performed by 1D and 2D nuclear magnetic resonance (NMR) spectroscopy and compared with previously published data. Although NMR is a most efficient method for the complete structural elucidation of natural products,^{6,7} a relatively large amount of pure sample is required. This involves time-consuming and costly isolation procedures or the use of expensive and complex (LC)/NMR instruments, both of which can considerably increase the identification costs.⁸ Mass spectrometry (MS), especially tandem mass spectrometry (MS/MS), has become one of the most important methods for the identification of trace natural products due to its high sensitivity and short analysis times. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) using collision-induced dissociation (CID) is a highly useful tool for the analyses of thermally labile substances (i.e. molecules with low vapor pressures and/or highly polar chemical functionalities).⁹ However, to fully characterize a compound from its MS/MS data (i.e. dereplication or other metabolomic approaches), a wide knowledge of the fragmentation pathways of homologous or congeneric compounds exhibiting a conserved structural core is required.^{10,11} Due to the few published studies on the ESI-MS/MS

fragmentation of diterpenes, further study is required.¹² Thus, ESI-MS/MS was employed here for the structural characterization of isopimarane diterpenes from Velloziaceae by a fragmentation approach using a quadrupole time-of-flight (QTOF) mass spectrometer and theoretical quantum chemistry calculations, when necessary.

Zhou and coworkers have previously proposed ESI-MS/MS fragmentation mechanisms for a series of *ent*-kaurane diterpenes isolated from the genus *Isodon*.¹³ Based on this, the aim of this study is to evaluate the gas-phase fragmentation profile and chemical behavior of a series of protonated isopimarane diterpenes (Figure 1).

Experimental

Materials and Isolation of diterpenes

Isopimarane diterpenes were selected for the present study, after initial analysis by gas-chromatography coupled to mass spectrometry with electron ionization source (GC/EI-MS) and also by GC using a flame ionization detector, to confirm the purity to be greater than 99% for all compounds¹⁴. Compounds **1**, **4** and **5** were obtained by the reduction of natural α,β -unsaturated carbonyl isopimaranes previously isolated from *Vellozia compacta*, Velloziaceae (collected at Serra do Cipó, Minas Gerais) with either Zn/acetic acid or by a Birch reduction using Na/NH₃.

Compounds **2** (Compactol) and **7** (11 β -Hydroxy-7-oxo-pimar-8(9)-15-diene) were isolated from a apolar extract of the stems, roots and leaf sheets of *Vellozia compacta*, Velloziaceae (collected at Serra do Cipó, Minas Gerais) by use of silica gel open column chromatography, as described in Pinto *et al.*¹⁵

Compounds **3** (methyl 7 β ,8,14 β -trihydroxy-15-isopimaren-18-oate) and **6** (methyl 8-hydroxy-7-oxo-15-isopimaren-18-oate) were isolated from an ethyl acetate extract of stems, roots and leaf sheets of *Vellozia pattens*, Velloziaceae (collected at Serra do Cipó, Minas Gerais) by use of silica gel open column chromatography, as described in Pinto *et al.*¹⁶

Compound **8** (11 β -Hydroxynanuzone) was isolated from the hexanic extract of stems, roots and leaf sheaths of *Vellozia nanuzae*, Velloziaceae (collected at Serra do Cipó, Minas Gerais) by use of silica gel open column chromatography, as described in Pinto *et al.*¹⁷

Compound **9** (13-Hydroxy-15,16-*bis-nor*-isopimaran-20,8-olide) was isolated from an ethyl acetate extract of stems and leaf sheaths of *Vellozia bicolor*, Velloziaceae (collected at Diamantina, Minas Gerais) by use of silica gel open column chromatography, as described in Pinto *et al.*¹⁸

Mass spectrometry analysis

Individual diterpene solutions were prepared at 0.01 mg/mL⁻¹ in acetonitrile/water (9:1). Samples were introduced into the ESI source by syringe pump and analysed by an ultrOTOF-Q (Bruker Daltonics, USA) mass spectrometer. Accurate-mass analyses was obtained by using a solution of sodium trifluoroacetic acid [(TFA)_n+Na]⁺ as internal standard. In order to improve protonation, a small quantity of formic acid was added to the solutions immediately prior to ESI-MS analysis. Analyses were performed under the following conditions: drying gas temperature, 160 °C, capillary voltage, 4500 V. Mass spectra were measured from *m/z* 90-400 in the positive ion mode. ESI-MS/MS analyses were performed by collision-induced dissociation (CID) using nitrogen as the collision gas with the collision energy varied in the range from 5 up to 25 eV for each precursor ion.

Computational Methods

In order to obtain the most stable conformers to these diterpene molecules, their geometries were submitted to conformational analysis using the MM2 force field.¹⁹ These compounds had their geometries re-optimized on the basis of the density functional theory (DFT) calculations and the calculated energies were obtained at B3LYP/6-31+G(d,p)²⁰ level of theory using Gaussian 03 software.²¹ The B3LYP/6-31G(d) model has previously been used in studies involving mass spectrometry and diterpenoids and the results are consistent with experimental measurements.²² Recently, this type of DFT calculation was used to distinguish between the intermediates of terpene

isopimaranes and the results obtained were used to increase the understanding of the biosyntheses for these compounds.²³ Determination of the protonation site was performed by calculation of the gas-phase basicity (GB) for each possible protonated site, by using the reaction between the neutral molecules with a proton.^{24,25}

Results and discussion

ESI-MS results

ESI-MS analysis of individual diterpenes showed the dissociation of some key functional groups even in the ionization source (source dissociation). These in-source fragment ions could be used as diagnostic ions, e.g., to identify the presence of hydroxyl groups, esters and carbonyl groups. On the other hand, the use of source dissociation ions as evidence of structural elucidation/characterization may easily lead to errors from less experienced mass spectrometry operators as it assumes that the sample is totally pure and that the ions are not due to the presence of another compound.^{26,27} We have opted to describe some of the ions observed during the ESI-MS source dissociation of isopimarane diterpenes, but we must stress, that for the reasons described above, these results must be used with considerable caution. MS/MS is able to provide more specific information about the skeleton of these compounds and is more reliable as the precursor ions are totally isolated in the gas-phase by the mass spectrometer prior to fragmentation.

ESI-MS analysis showed that the fragment ion resulting from the loss of water $[M+H-H_2O]^+$ had the highest intensity for the diterpenes **1**, **2**, **7** and **9** (see table 1). Ions arising from the loss of a second molecule of water, for instance for the di-hydroxylated diterpene, as well as the presence of protonated and cationized molecule were also observed. However, they were observed in lower relative abundance when compared to the dehydrated molecule. From these results, it is possible to conclude that for compounds that exhibit two hydroxyl groups, there are successive losses of mass 18 which can be attributed to H₂O losses.

The presence of an ester group can lead to the loss of the corresponding acid or ester (depending on the structure). ESI-MS studies of sesquiterpene lactones indicate that the side chain elimination can occur for an ester, which could be used to characterize the various metabolites from MS studies.²⁸ In the present study, the analysis of diterpenes **3** and **9**, showed loss of methyl formate, formic acid (46 u) and acetic acid (60 u), respectively. Table 1 shows the ions observed from the ESI-MS (source dissociation) analysis and their relative intensities.

A very intense fragment ion is observed for loss of water (90% relative intensity) for compound **3**. Like the other diterpenes, this ion subsequently loses methyl formate to generate a low intensity ion. A different behavior was observed for the diterpenes **4**, **5** and **7**, which as well as having a hydroxyl group, have a ketone in the structure. The most intense ions were observed for the protonated molecule $[M+H]^+$. This is indicative of protonation occurring on the relatively basic carbonyl group leading to a more intense protonated ion when compared to hydroxylated only molecules or that the ion is more stable with no in-source dissociation ions observed

For diterpenes **4**, **5**, and **7**, the most intense ion in the ESI-MS spectra (Table 1) was the cationized molecule. Diterpene **6**, which also has one hydroxyl and one keto group, shows different behavior again. The loss of water is the most intense ion in the mass spectrum and the corresponding protonated molecule is only observed at about 40% relative abundance. All these observations can be carefully taken by the strong influence of the source voltage. For this reason, we performed the comparison with the same source settings.

ESI-MS/MS profile of $[M+H]^+$ molecules and computational studies

Analysis of nine diterpenes was performed by ESI-MS/MS. The protonated molecule was selected as the precursor ion and dissociated by CID-MS/MS. The results are summarized in Table 2 and Fig. 2 (all the mass spectra can be found in the Supporting information).

The MS/MS spectra of **1** and **2** ($E_{lab} = 10$ eV) show the product ion m/z 289 as the base peak, which is proposed to be due to the loss of water from the precursor ion (i.e. $[M+H-H_2O]^+$). The presence of

hydroxyl groups provides the possibility of H₂O loss via two possible processes: (i) by the charge-remote elimination reaction or (ii) by charge driven concerted reaction. In the previous EI studies of isopimaranes, the loss of 18 u was attributed to a charge-remote E2 elimination reaction.²⁸ However, for protonated molecules, the identification of fragmentation pathways is not so simple, because the proton influences the electron density after protonation and, consequently, the driving force to form more stable ions.

The application of computational chemistry, in order to clarify and identify the protonation sites and fragmentation mechanisms, has been widely used, and the results obtained are largely in agreement with experimental observations. In this sense, the proton affinities (PA) and gas-phase basicities for the diterpenes were calculated and the results are summarized in Figure 3. The use of DFT calculations has been demonstrated to be useful to understand the fragmentation and biosynthesis of isopimaranes.²²

Firstly, the protonation sites are indicated by analyzing the molecular electrostatic potential map (MEP) and Mulliken-Kohn-Sigman (MK) atomic charge that suggest the most reactive sites for protonation, see Figure 4. The use of MEP and atomic charge analysis improves the reliability of the calculation of the protonation site. For the isopimarane diterpenes in the present study, MEP indicates that protonation takes place at the oxygen atoms. This is in agreement with the MK atomic charges. However, this contribution is not decisive to suggest the protonation site. The strongest indicator is the proton affinity (PA) and gas-phase basicity (GB), which describe the ions formed. The PA values are shown in Fig. 3.

Protonations for compounds **1** and **2** take place on the hydroxyl group – in agreement with the reactivity for alcohols. PA values for these compounds exhibit small differences for protonation at oxygen atoms, which suggests that protonation can take place in any of the alcohol positions. For **2**, a difference between the calculated PA values is observed because protonation of the vicinal oxygen leads to formation of a hydrogen bond, resulting in a structure without geometry differences (Fig. 3). Scheme 1 shows the fragmentation proposed for isopimaranes **1** and **2**.

In the MS/MS analysis of protonated **1** and **2**, the main product ions may result from two consecutive losses of 18 u. The first loss of 18 u occurs in the same intensity for both compounds, as observed in the MS/MS spectra at $E_{\text{lab}} = 10$ eV. The protonation on the hydroxyl group leads to loss of 18 u by a drive charge mechanism. However the PA values indicate that compound **2** is most basic than **1** (Fig. 3). The proximity between –OH for compound **2** can induce the two H₂O eliminations, after collision, when compared with computational results. The same conclusions can be attributed to **3**, which has three water losses forming the product ions m/z 349, 331 and 313. Reaction enthalpy calculations (using the B3LYP/6-31+G(d,p) theory set) indicates that the first water loss for protonated **1**, **2** and, **3** occurs via a charge-induced dissociation. From an analytical viewpoint, the consecutive H₂O eliminations may be used to suggest the presence of –OH group in the isopimarane core.

For compounds **4**, **5** and **6** protonation takes place on the carbonyl group. The fragmentation proposed for the compound **5** (Scheme 2) can also be assigned to compounds **4** and **6**. Compound **7** exhibits the same behavior as **3**, with protonation on carbonyl occurring at low intensity (see Fig. 3 for PA values). Compounds **4** and **5** generate product ions that have the same m/z values (see Table 1 and Fig. 2), however, the formation of m/z 269, due to two losses of water, for compound **4** and the presence of m/z 163 for compound **5** can be used as diagnostic ions.

Calculation of PA values of compounds **7** and **8** suggest that protonation occurs on the carbonyl group. This is also backed up by evidence from other combined mass spectrometry and computational studies of the ketones and butenolides.²⁹⁻³³ Protonation on the α,β -carbonyl leads to multiple losses of water,³³⁻³⁴ as suggested by Scheme 3. The possibility of water losses from the protonated precursor ion forms the stable ions at m/z 285 and m/z 301, respectively for compounds **7** and **8**.

The ESI-MS spectrum of diterpene **9** (Fig. 5) exhibits a product ion m/z 275 as the base peak, which results from the loss of H₂O from the protonated molecule at m/z 293. The ions at m/z 247 and 229, results from the loss of formic acid and water plus formic acid respectively. These occur at much low relative intensity. In the ESI-MS/MS analysis (m/z 293 as the precursor ion) the product ion m/z 275 is the most intense ion whilst m/z 247 is the least intense. Fig. 2 (ESI-MS/MS spectrum of [**9** + H]⁺

shows the energy resolved pots of the protonated molecule. With increasing collision energy, the product ion m/z 275 dissociates to generate a product ion at m/z 229, resulting from the loss of formic acid. The ESI-MS/MS spectrum of the protonated molecule $[M+H-H_2O]^+$ m/z 275 led to the product ion m/z 229 as being the most abundant even with increasing collision energy.

Early studies with lactones show that the protonation takes place at the carbonyl and the collision can induce to proton migration and ring opening.³²⁻³⁴ This mechanism is suggested by consecutive H_2O and CO losses.**Error! Bookmark not defined.** In the present study, the PA calculations suggest that the protonation occurs on the lactone ring. Therefore, it is proposed that the main fragmentation mechanism must occur by ring opening and loss of H_2O to form the product ion m/z 275. The high intensity of this product ion suggests the possibility of the hydroxyl at position 13 having a contributing affect. Scheme 4 shows the proposed fragmentation by two routes. The first route is the primary one occurring from the protonation of the carbonyl oxygen, leading to the formation of an acylium ion, which can undergo losses of H_2O and CO, to form product ion m/z 247. In the second route, the water loss occurs initially at position 13.

Conclusions

The studies with natural diterpene isopimaranes by ESI-MS showed that the intensity of protonated and cationized molecules could be used to distinguish these compounds. ESI-MS/MS analyses for the protonated compounds show that the intensity and number of fragments ions were important for characterization. Multiple losses of water indicate the number of hydroxyls in the isopimarane core structure and the relative intensities of the resulting product ions can aid the characterization of congeners. The fragmentation mechanisms are very similar, with the most intense product ions occurring through Retro-Diels-Alder (RDA) reactions or water losses via charge-remote reactions. This information is important as it enables us to distinguish these molecules from their isomers. Computational results corroborated with ESI-MS and ESI-MS/MS studies where the protonation site was shown to change with each compound analyzed. The results from this work will aid the

characterization of new and novel compounds as well as biosynthetic studies of isopimarane diterpene derivatives.

Supplementary Information

Supplementary data is available on line. All the mass spectra can be assessed in the supporting information. The computational results are available from corresponding authors.

Acknowledgements

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Table 1. Ions m/z observed by ESI-MS (relative abundance in parentheses).

Compound	MM	[M+Na] ⁺ and [M+K] ⁺	[M+H] ⁺	[M+H-H ₂ O] ⁺	[M+H-2H ₂ O] ⁺
1	306	329 (17)	307 (35)	289 (100)	271 (7)
2	306	329 (43)	307 (21)	289 (100)	271 (14)
3	366	389 (100) and 405 (38)	367 (12)	349 (91)	331 (25)
4	304	327 (12)	305 (100)	287 (43)	
5	304		305 (100)	287 (25)	
6	348	371 (24) and 387 (12)	349 (41)	331 (100)	313 (9)
7	302	325 (19)	303 (100)	285 (51)	
8	318		319 (89)	301 (100)	283 (36)
9	292	315 (19)	293 (47)	275 (100)	

Table 2. Major product ions (m/z), with relative abundances in parenthesis, from the ESI-MS/MS analyses of the protonated precursor ions of the compounds **1–10** (at 10 eV).

Compound	[M+H] ⁺	MS/MS
1	307	289 (100); 271 (15); 165 (11)
2	307	289 (100); 271 (40); 169 (13); 165 (13)
3	366	349 (33); 331 (33); 313 (27); 289 (20); 271 (100); 253 (47)
4	305	305 (21); 287 (100); 269 (5); 181 (42); 109 (16)
5	305	305 (100); 287 (75); 181 (13); 163 (13) PB; 109 (6)
6	349	331 (36); 299 (36); 289 (14); 271 (100); 253 (50); 181 (14)
7	303	303 (28); 285 (100); 267 (7); 257 (6); 243 (6); 229 (14); 179 (28)
8	319	319 (29); 301 (78); 283 (100); 273 (87); 261 (58); 161 (48); 123 (38)
9	293	275 (14); 247 (7); 229 (100)

Figures

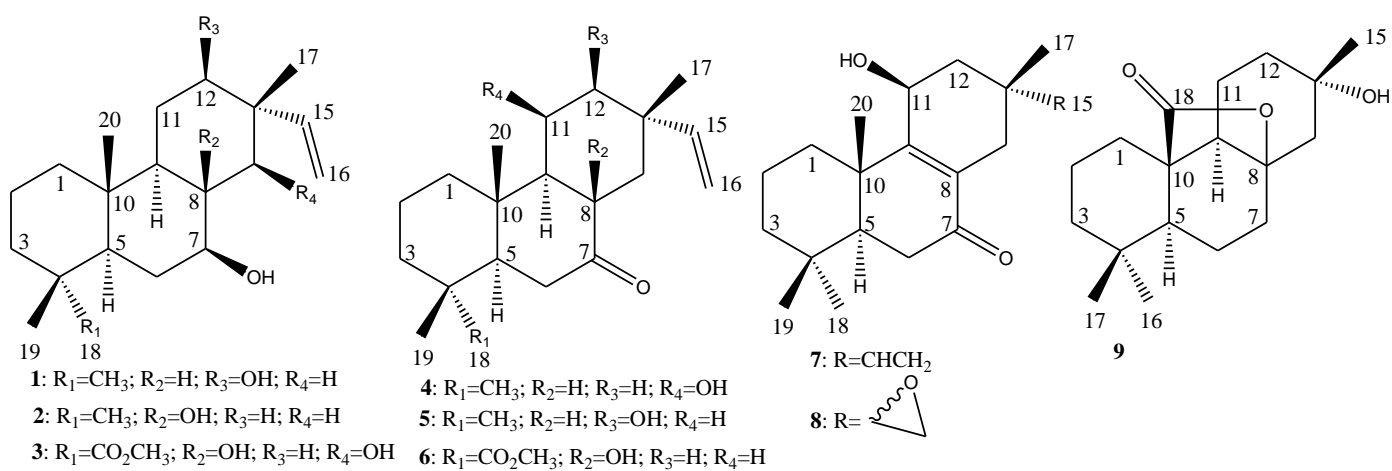
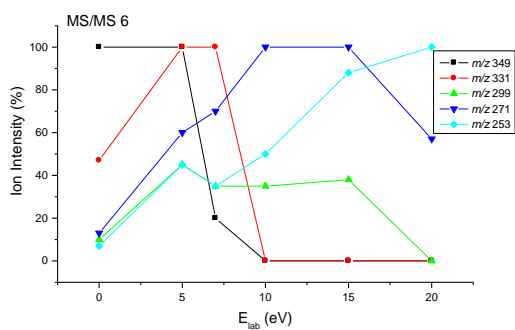
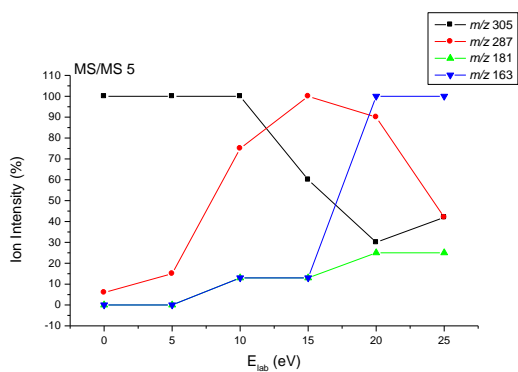
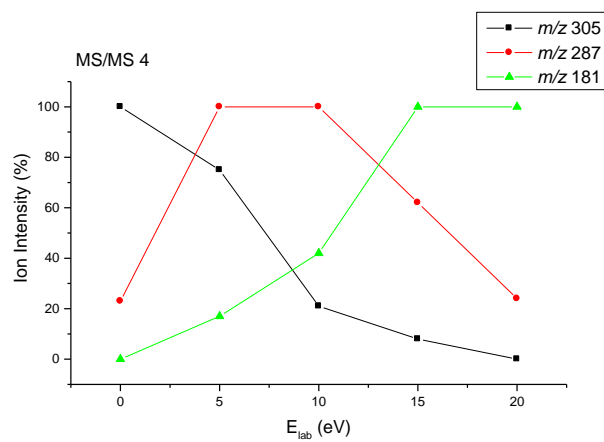
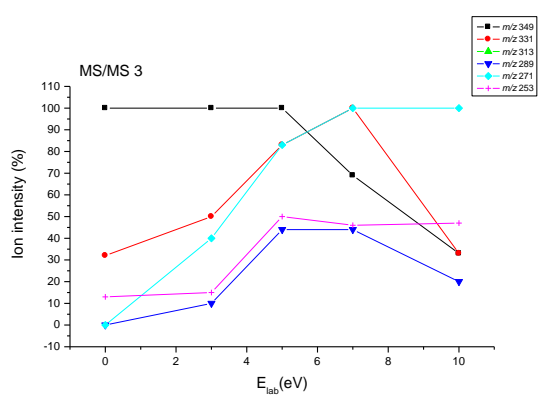
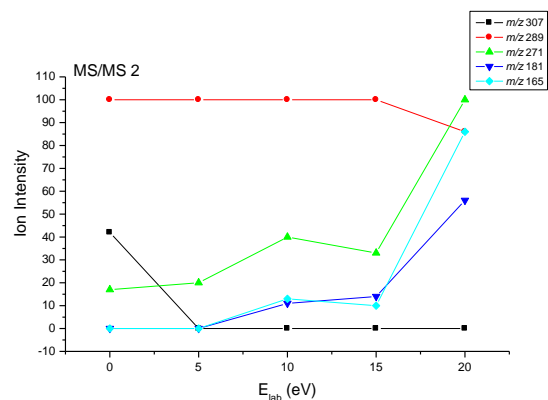
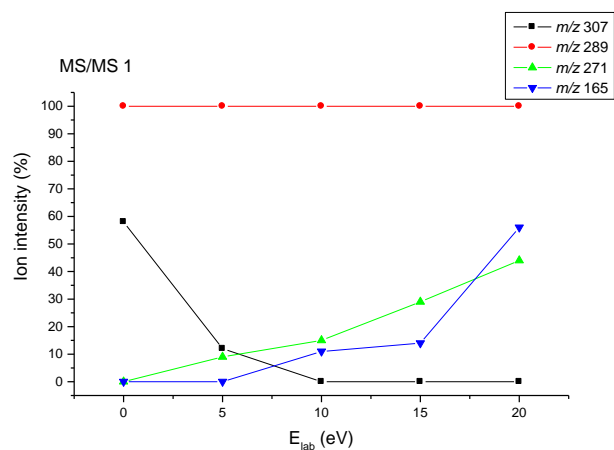


Figure 1. Structures of isopimarane diterpenes isolated from Brazilian species of *Vellozia*.



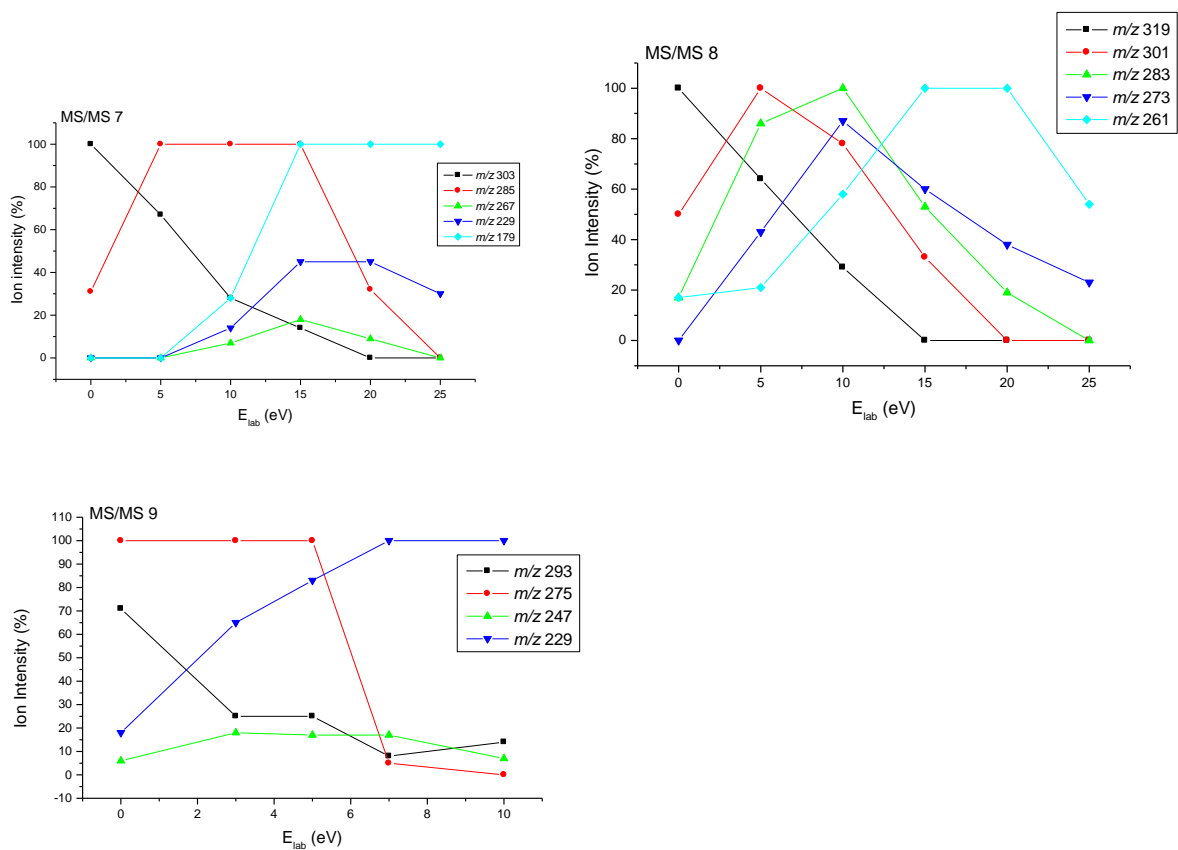


Figure 2. Energy-resolved plots of protonated isopimarane diterpenes. All the ESI-MS/MS can be assessed at supporting information.

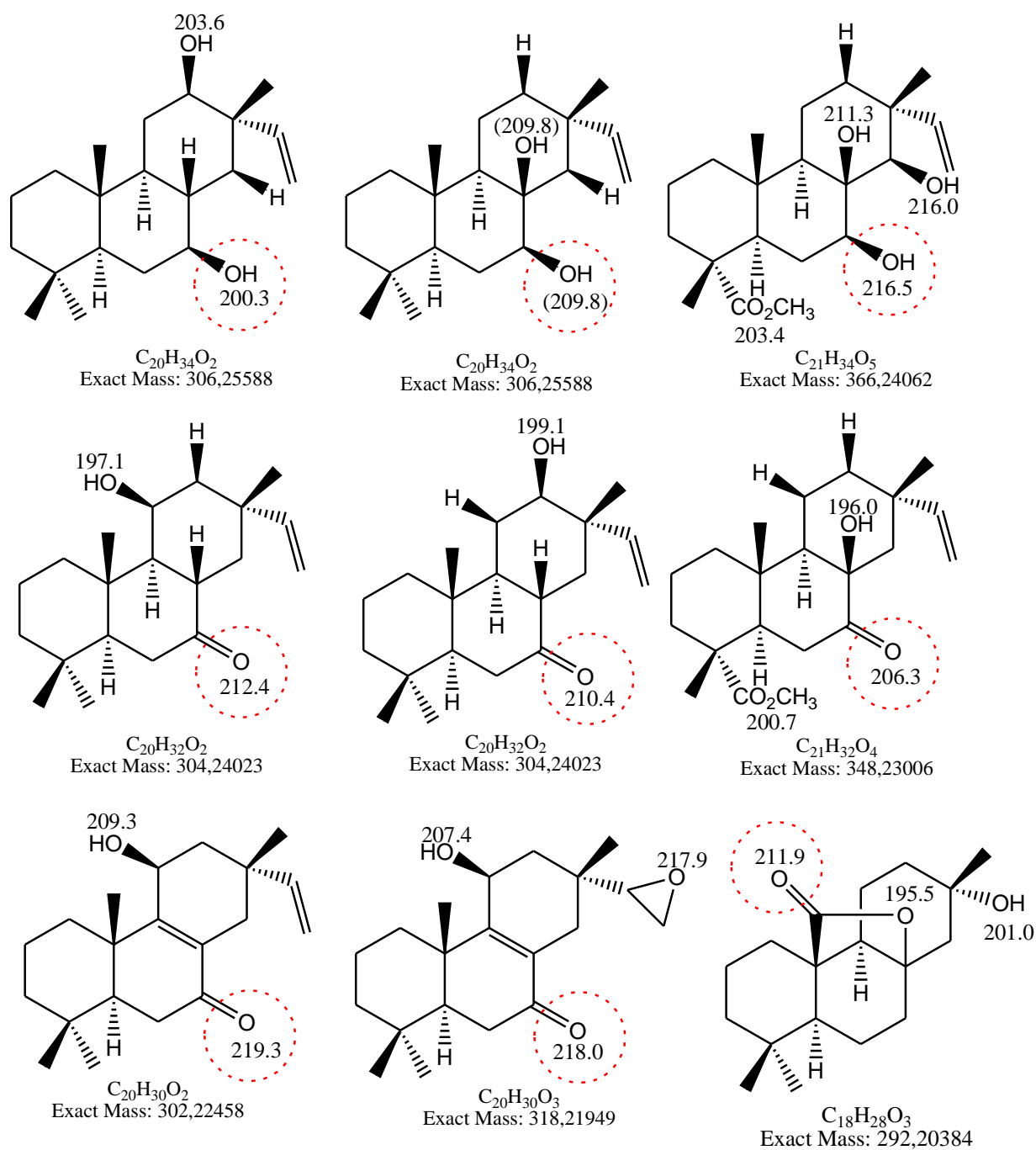


Figure 3. Proton affinities (PA), exact masses, and molecular formulas for isopimaranes 1-9. The PA were calculated at B3LYP/6-31+G(d,p) level. All the values for PA are in kcal.mol⁻¹. The red circle indicates the protonation site.

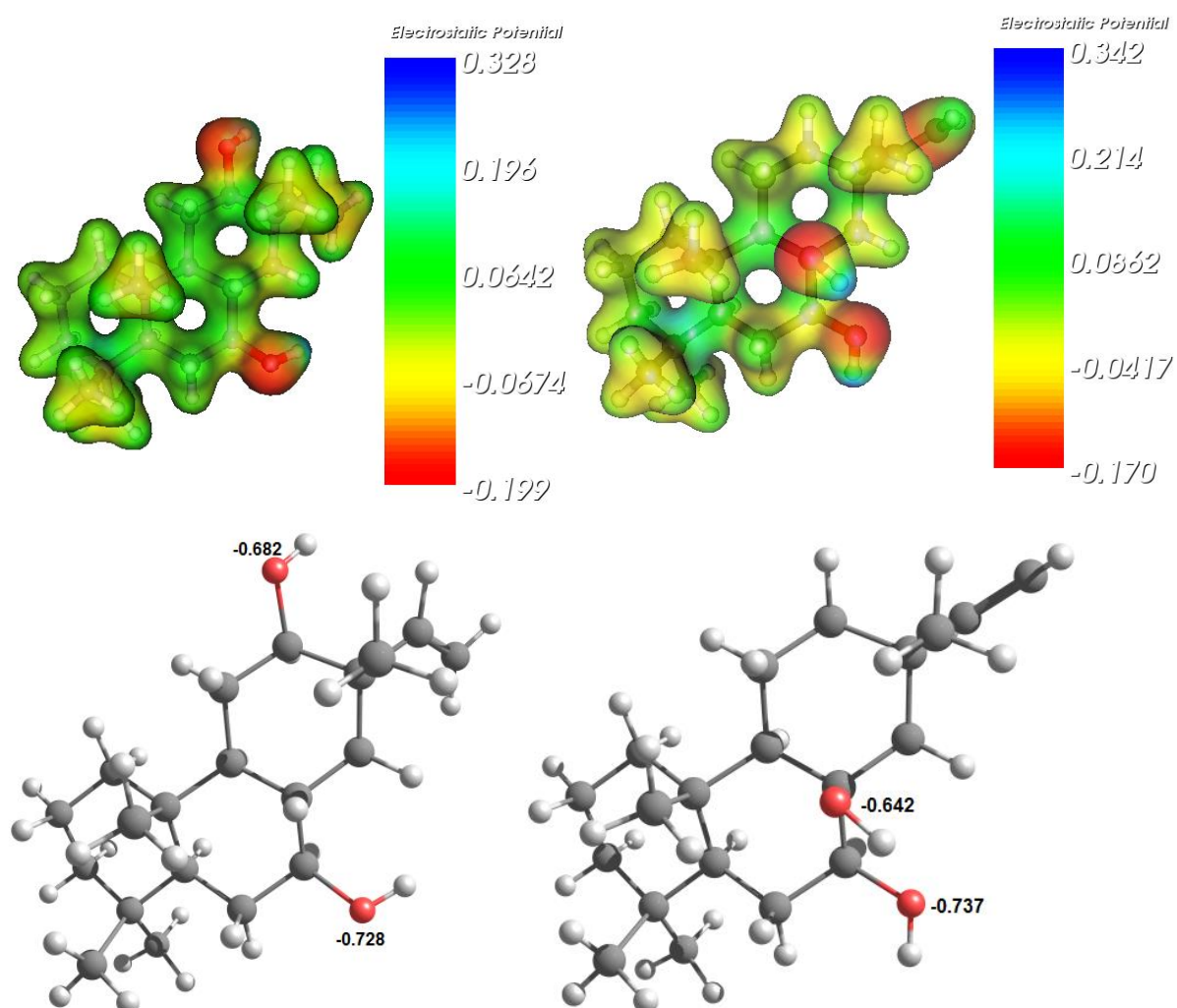
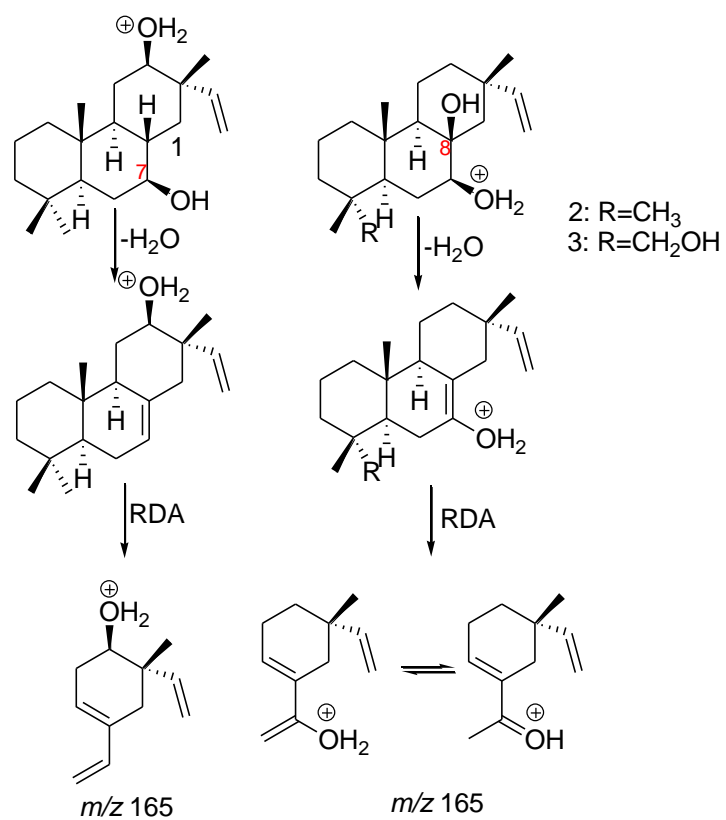
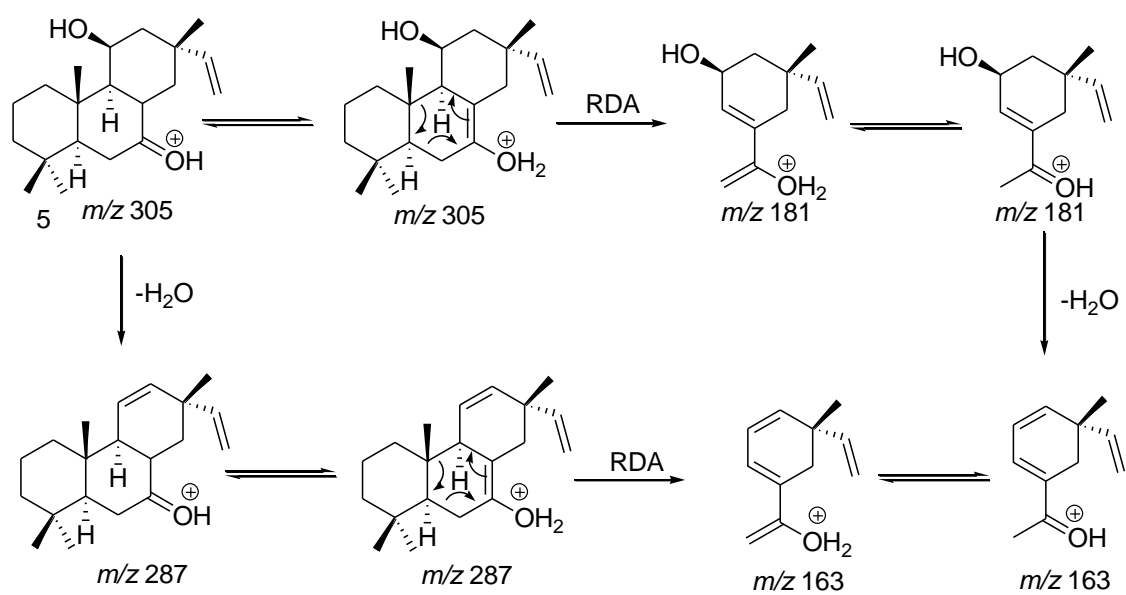


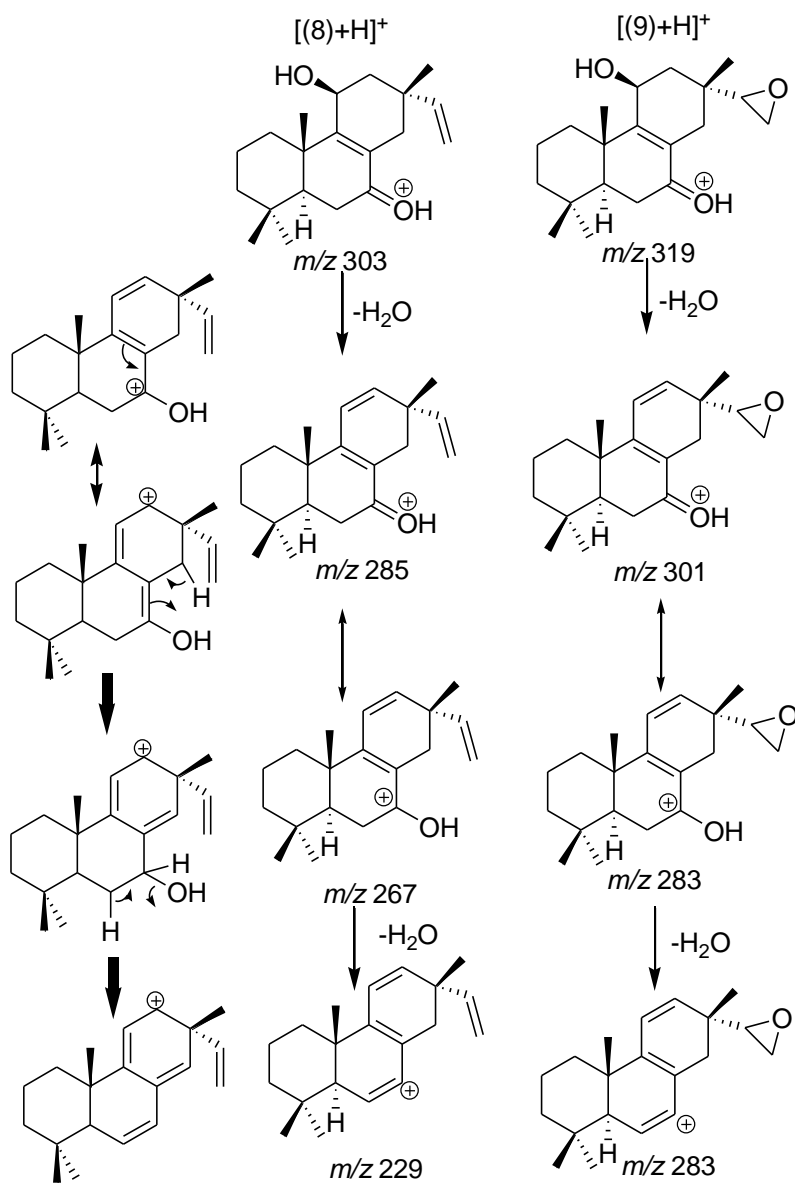
Figure 4. MEP and MK atomic charges (in electron unities) for isopimaranes **1** and **2** calculated at B3LYP/6-31+G(d,p) level.



Scheme 1. Proposed fragmentation for isopimaranes **1** and **2**. The protonation sites were suggested on the basis of the computed proton affinities displayed at Figure 3.



Scheme 2. Proposed fragmentation for isopimarane **5**.



Scheme 3. Proposed fragmentation of protonated **7** and **8**.

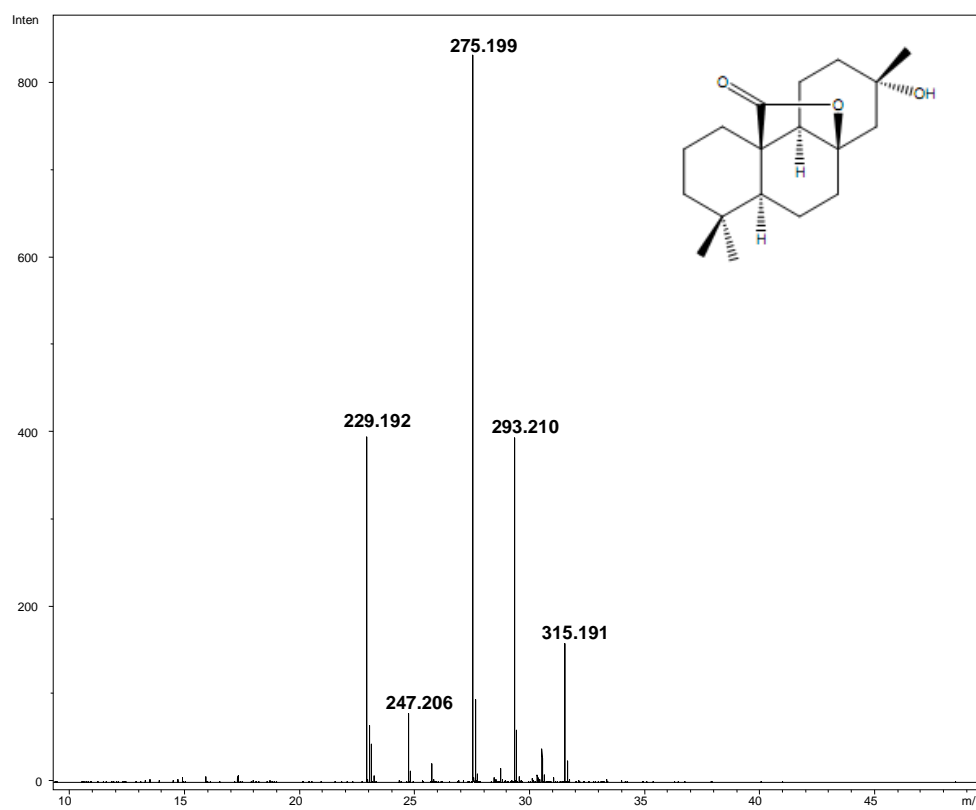
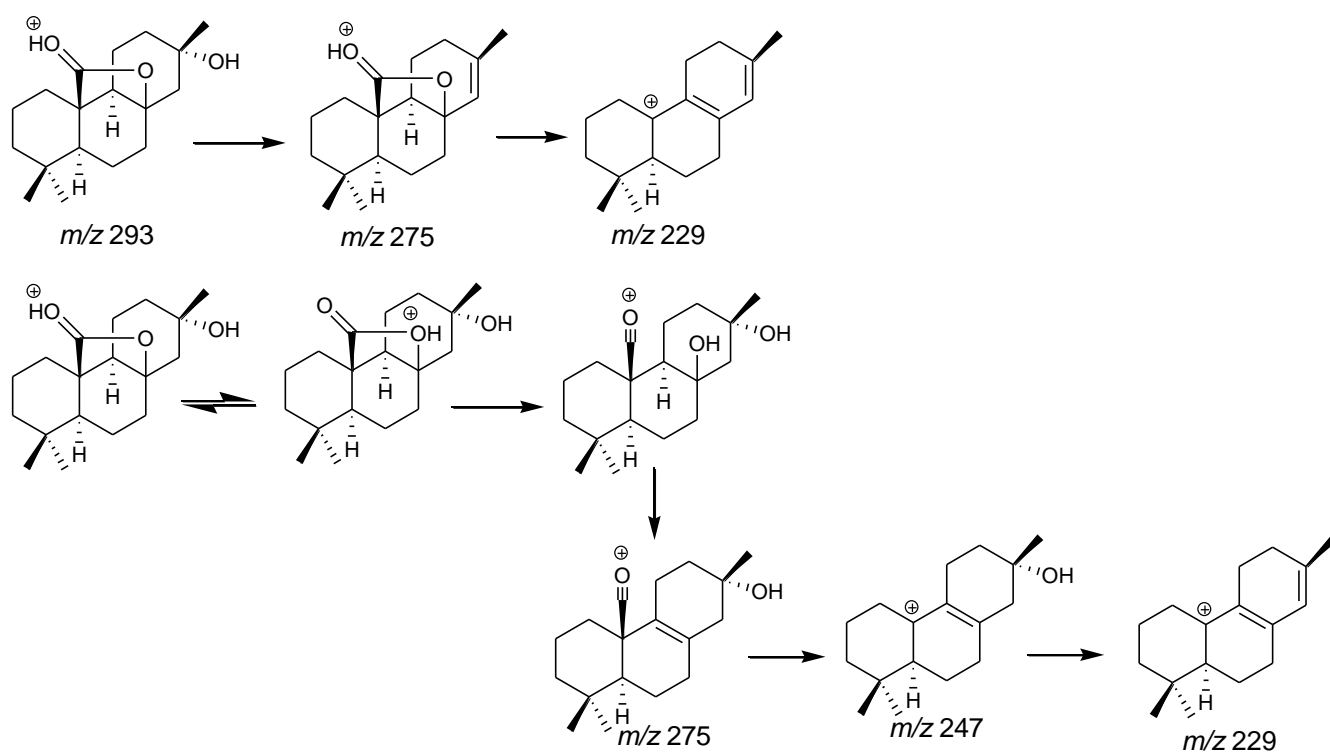


Figure 5. Low resolution ESI-MS/MS spectrum in the positive mode for $[\mathbf{9}+\text{H}]^+$.



Scheme 4. Proposed fragmentation of protonated **9**.

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